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EXPRESSION OF PML TUMOR SUPPRESSOR IN A431 CELLS REDUCES CELLULAR GROWTH BY INHIBITING THE EGFR EXPRESSION*

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Abstract – Our previous studies showed that the promyelocytic leukemia protein, PML, functions as a cellular and growth suppressor. Transient expression of PML was also found to repress the activity of the epidermal growth factor receptor (EGFR) gene promoter. In this study, we have examined the effects of PML on A431 cells, which express a high level of EGFR protein. The *PML* gene was introduced into the cells using the adenovirus-mediated gene transfer system. Western blot analysis on the extracts from the cells expressing PML showed a significant repression in the expression of the EGFR protein. The cells were examined for growth and DNA synthesis. The data showed a marked reduction in both growth and DNA synthesis rate in the cells expressing PML compared with the control cells. Furthermore, in comparison with the controls, the cells expressing PML were found to be more in G1 phase, fewer in S and about the same number in the G2/M phase. These data clearly demonstrated that the repression of EGFR expression in A431 cells by PML was associated with inhibition of cell growth and alteration of the cell cycle distribution, suggesting a novel mechanism for the known growth inhibitory effects of PML.

Keywords – PML, EGFR, cell cycle, A431 cells, expression

1. INTRODUCTION

PML belongs to a family of nuclear proteins which contain several functional domains including the RING-finger, two cys/his rich regions designated as the B box motifs and an α-helical domain, which is involved in PML/PML dimerization [1-4]. PML is the major component of a novel nuclear body named PML oncogenic domain (POD) [5]. Many proteins co-localized with POD have been identified including SP100, the ubiquitin-like protein modifier, SUMU-1/PIC-1/sentrin, the interferon induced protein ISG20, and immediate early viral proteins IE1 and IE4 [6-8]. POD was found to be the site of viral DNA replication and transcription. Nascent RNA polymerase II transcripts were found within the POD, and that PML co-localized with the transcription co-regulator CBP (CREB binding protein) [9]. These findings support the notion that PML may be involved in transcription regulation.

The transcription regulatory function of PML has been demonstrated in several of our previous studies [10-13] and others [14]. These studies showed that PML functioned as promoter dependent transcription activator and inhibitor. PML could activate the transcription of a steroid hormone receptor [14] and transcription mediated by Fos/AP-1 [10]. However, when fused to the GAL4 DNA-

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binding domain, PML acted as a transcription repressor to inhibit transcription from the GAL4-responsive promoters [11]. Specific domain of the PML protein was involved in the transcription repressing events. Furthermore, our studies showed that PML suppressed the promoter of the epidermal growth factor receptor (EGFR) gene by inhibiting EGFR's Sp1-dependent activity. In the study, it was shown that PML physically and functionally interacted with SP1 [13]. Recently, we reported that the inhibitory effects of PML on transcription were due to its association with histone deacetylases and their recruitment to the promoter [12].

There are many reports that PML is a cellular growth and transformation suppressor [14-18]. Transient expression of PML induced a G1 arrest and apoptosis in MCF-7 cells [15]. In HeLa cells, a stable expression of PML induced growth inhibition by lengthening the G1 phase of the cell cycle [17]. PML affects cell cycle progression by modulating the expression of several key proteins involved in the G1/S checkpoint and dephosphorylation of Rb was observed [17]. The *PML* gene knock-out study reported by Wang et al. [19] strongly support a crucial role for PML in the control of cell growth. Recent studies demonstrated that PML is essential for multiple pathways of program cell death by using $PML^{-/-}$ mice and in cells overexpressing PML [20]. Interestingly, POD recruits several important cell cycle regulators (e.g. BAX and p27KIP1) to the nucleus during induction of apoptosis [21].

EGFR mediates the effects of epidermal growth factor (EGF) on cell growth [22-23]. The high level of expression of EGFR has been reported to be associated with a number of malignancies including breast, ovarian and clone cancer [24-25]. Given the demonstrated role of PML in suppressing cellular growth, and its inhibitory effects on the EGFR promoter activity, it was, therefore, necessary to examine whether the expression of EGFR protein could be suppressed by PML. For this purpose, using the adenovirus-mediated gene transfer system, PML was expressed in A431 cells, which overproduce EGFR protein. The results showed that a high level expression of PML suppressed the EGFR expression at 60 hours post-infection, whereas control adenovirus (without PML) had no effect on EGFR expression. Furthermore, in the cells expressing the PML protein, the growth and DNA synthesis showed a significant reduction followed by alteration in the cell cycle progression.

2. MATERIALS AND METHODS

a) Cell culture and transfection

A431 cells were maintained in Dulbeccos modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO/BRL, Gaithersburg, MD) in 5% CO_2 at 37 ˚C in a humidified incubator. The recombinant PML adenovirus (Ad/PML), the antisense PML adenovirus (AdAs/PML) and the adenovirus (Ad) vectors were constructed and produced as previously described [26]. The average titer of the viruses used in this study was 5×10^5 plaque forming unit (pfu)/ml. For infections, cells were plated at 5×10^4/60 mm tissue culture dishes. 2 μl of the viruses were used for each infection. The experiments were performed in triplicate.

b) Western blot analysis

The expression of PML protein in A431 cells was evaluated using Western blotting as described in our previous report [13]. Briefly, after appropriate culture times as indicated in the legend to the Figures, cells were washed and harvested in phosphate buffer saline (PBS) and lysed. Total cell extract was then prepared. Proteins in the samples were resolved on 10% SDS-polyacrylamide gel (SDS-PAGE), and then

transferred onto nitrocellulose membrane. The membrane was probed with anti-PML, anti-EGFR, and/or anti-actin antibodies as appropriate. Antibodies used were all polyclonal, which were prepared from rabbits. The anti-EGFR, and anti-actin antibodies were from Santa Cruse Biothech., USA, and the anti-PML was raised against a GST-PML fusion protein in our laboratory [18].

C) Flow cytometric analysis

For analysis of DNA content by flow cytometry, cells from exponential cultures were fixed for 24 hrs in 70% ethanol, washed in PBS three times and resuspended in PBS containing 50 μg/ml propidium iodide (Sigma Chem. Co.), 20 μg/ml DNase free RNase A. Cells were left in the dark overnight before being analysed on a FACSCAN flow cytometer equipped with double discriminator and LYSYS II and CELLFT software (Becton-Dickinson).

d) In vitro growth rate assay

The *in vitro* growth rate of A431 cells infected with adenovirus vectors was assessed using the trypan blue exclusion assay as described previously [17]. For this assay, A431 cells were plated at 1 X 10^4 cells /well in 24-well plates. The number of the cells was determined at 24 hr intervals as shown in the Figures.

e) [^{3}H] thymidine incorporation assay

The DNA synthesis rate of A431 cells which reflect cell proliferation was determined using the [^{3}H] thymidine incorporation assay. Cells were plated at 1 X 10^4 cells/plate in 24-well plates and cultured in complete medium. Cells were then infected with the adenovirus vectors. [^{3}H] thymidine (2 mCi) was added to each well at 24, 48, and 72 hours, and the cells were incubated for an additional 2 hours at 37 ˚C. Cells were then washed in PBS and harvested as described previously [17]. Radioactivity of the incorporated [^{3}H] thymidine was determined in a scintillation counter.

3. RESULTS AND DISCUSSION

a) Suppression of EGFR expression by PML in A431 cells

Epidermal growth factor (EGFR) receptor mediates the effect of the epidermal growth factor (EGF), playing an important role in the control of cell growth. Given the demonstrated role of PML in suppressing the cell growth and its inhibitory effects on the EGFR promoter activity, the effect of PML on the expression of EGFR protein was examined. In our previous study, PML was expressed in adenovirus vectors, which efficiently infect animal cells and result in a high level expression of the PML protein [26]. To examine the effects of PML on EGFR expression, the *PML* gene was introduced into the A431 cells using the adenovirus-mediated gene transfer system. A431 cells express a high level of the EGFR protein, which can be easily detected by western blotting. Following infection, the cells were cultured for different periods of time and then harvested, and total cell extracts were prepared (see Materials and Methods). Total proteins from A431 cells expressing high levels of PML (Ad-PML) and cells infected with control adenovirus (AdAs/PML) were prepared at the time courses indicated in Fig. 1. The proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane and probed with anti-EGFR or anti-PML antibody. As shown in Fig. 1A, examination of the cell extracts for the PML protein expression using an anti-PML antibody, indicated that PML is efficiently expressed in the infected cells, giving a band of 68 kDa which represents the PML protein [18]. The expression of the PML protein was found to be at the highest

level at 72 hours post infection of the cells. The cells infected with the control adenovirus vector containing the anti-sense PML (Ad-ASPML, PML in reverse orientation) did not express a detectable level of PML. It should be noted that the PML protein is normally expressed at a very low level in the cells. However, in A431 cells we could not detect the endogenous PML protein using our anti-PML antibody.

Having confirmed the expression of the PML protein in A431, the expression of the EGFR protein was examined using an anti-EGFR (Fig. 1B) antibody. In order to monitor the correct protein loading, the membrane was stripped of the anti-EGFR antibody and re-probed with an anti-actin antibody (Actin) (Fig. 1B). Analysis of the expression of the EGFR protein in A431 cells showed no significant change in the level of the protein up to 48 hours post infection. The anti-EGFR antibody detected a 170 kDa band representing the EGFR protein. However at 60 hours there was a sharp reduction in the expression of the EGFR protein. At this time, the level of the PML protein in the cells reached its highest level. The delay in the effects of the PML protein on the EGFR expression suggested that a threshold for the PML's effects might be present in the cell. Moreover, a competition between PML and factors regulating the EGFR gene promoter such as Sp1 might explain the effects of PML on the expression of EGFR (see below).

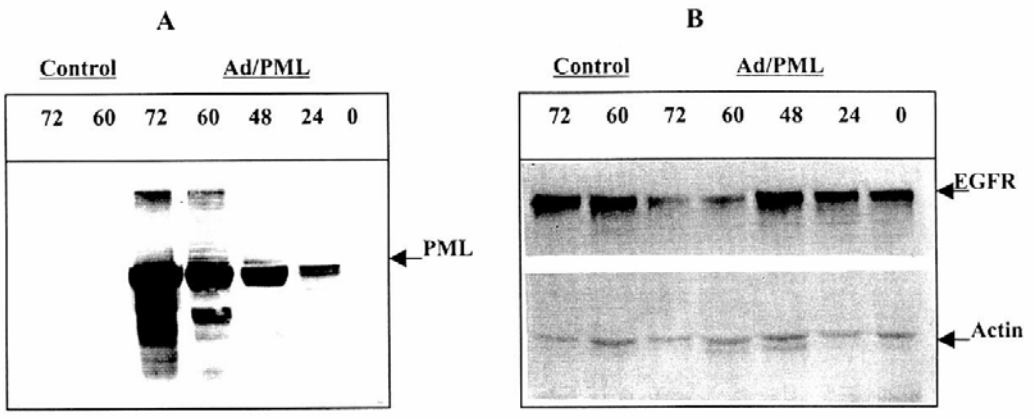

Fig. 1. Analysis of the expression of PML and EGFR in A431 cells
A) Transient expression of PML. Abbreviations are as follows: Ad/PML, adenovirus containing the *PML* gene; control cells infected with the control virus AdAs/PML. B) Analysis of the EGFR expression. The position of the EGFR and actin proteins is indicated by arrows. For details see materials and methods

Given the demonstrated inhibitory effects of PML on EGFR promoter [13], the repression of the EGFR protein expression by PML could be due to the inhibition of its promoter activity. The promoter activity of EGFR is mainly regulated by the Sp1 transcription factor [22]. Our previous findings showed that PML physically and functionally interacted with Sp1, disrupting the binding of Sp1 to the promoter [13]. *In vivo* and *in vitro* experiments demonstrated that the association of the Sp1 and PML had a significant effect on the EGFR promoter activity and transcription [13]. Furthermore, on northern analysis on the extracts from MCF7 cells infected with the Ad-PML using a probe to the 5' region of the molecule, a significant reduction in the endogenous expression of the EGFR mRNA was observed (data not shown). The repression of the EGFR's protein expression in the presence of PML confirmed our previous report, and indicated that PML could negatively regulate both transcription and expression of the *EGFR* gene promoter.

b) Expression of PML significantly reduces A431 cell growth

To investigate whether the repression of the EGFR expression by PML could alter the growth of A431 cells, the growth characteristics of the cells were analyzed in the presence or absence of PML expression. A431 cells infected with Ad/PML or AdAs/PML and the number of cells was determined at 24 hour intervals (see Materials and Methods). As shown in Figure 2A, in the cells expressing PML (Ad/PML), the growth rate had significantly reduced compared to the control cells (AdAs/PML). The effects of PML on cell growth were more prominent at 72 hours post infection. From this time point onwards, cells started to come off the culture and the number of viable cells were significantly (up to 4-fold) reduced. These data indicated that the expression of PML had a suppressive effect on the growth of A431 cells, and at very high concentrations can be lethal to the cells.

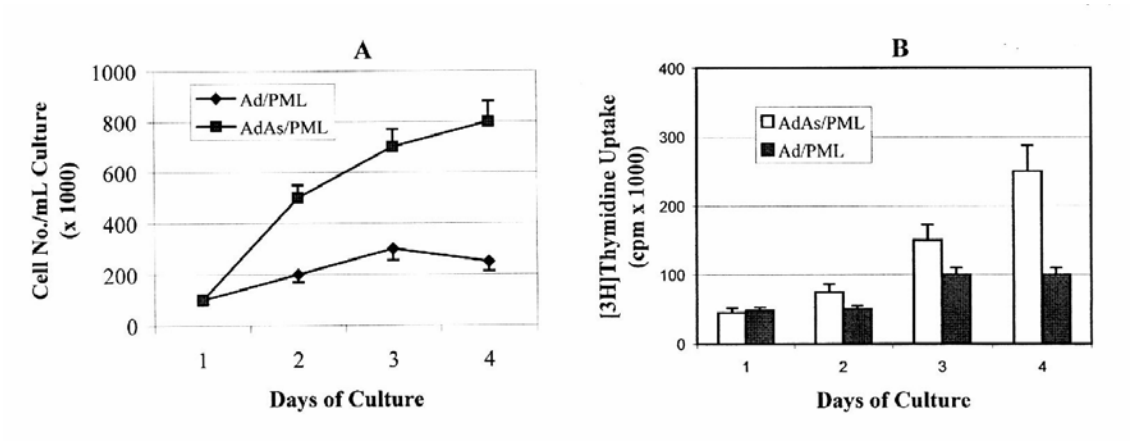

Fig. 2. Suppression of the growth rate of A431 cells by PML
A) Infection of A431 cells with Ad/PML and AdAs/PML. B) Incubation of A431 cells with [^{3}H] thymidine. The data are shown in count per minute (cpm). For abbreviations see legend to Fig. 1

To further investigate the effects of PML on the growth rate of A431 cells, the synthesis of DNA in these cells were examined in the presence of PML. Cells were infected with adenovirus vectors (Ad/PML and AdAs/PML) and incubated with [^{3}H] thymidine at 24 hr intervals. The incorporation of [^{3}H] thymidine into DNA was measured as an index for DNA synthesis. As shown in Fig. 2B, the cell expressing PML had incorporated a much lower [^{3}H] thymidine, compared to the control cells (AdAs/PML), suggesting a reduced DNA synthesis rate in the presence of PML.

c) Effects of PML on the cell cycle distribution of A431 cells

Since the significant effect of PML on cell growth (see above), we sought to investigate the distribution of cell cycle phases in the A431 cells in the presence of PML. In order to analyze the cell cycle distribution, a flow cytometry analysis was performed on the exponentially growing cells after expression of PML. As indicated in Figure 3, A431 cells expressing PML (Ad/PML) showed a proportionally high number of cells in G1 (73%), few cells in S (20%), and a very low number in G2/M (7%). In control cells, the distribution of the cell cycle was more uniform, so that about 55% of cells were in G1, 35% in S and 20% in G2/M phase. These data demonstrated that the expression of PML had lengthened the duration of the G1 phase of the cell cycle in A431 cells.

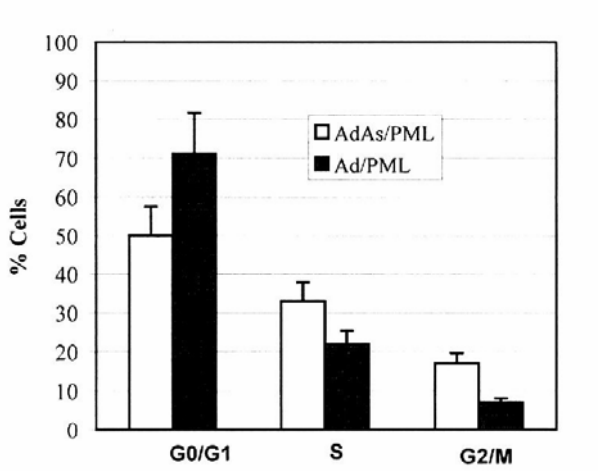

Fig. 3. Effects of PML on cell cycle distribution in A431 cells. Cell cycle distribution of the A431 cells in the presence (Ad/PML) and absence (Ad As/PML) of PML expression. For details, see materials and methods

On the basis of the above observations, it is suggested that PML is likely to be involved in cell cycle control, affecting factors which control the G1/S transition. In fact, it has been reported that the number of PODs is cell cycle dependent, with the highest number at the G1 phase. Furthermore, it has been demonstrated that PML is associated with the well-documented cell cycle negative regulator tumor suppressor and retinoblastoma protein, Rb [24]. The Rb protein functions as a master regulator of the G1/S checkpoint. It is likely that PML exerts its inhibitory effects on the cell cycle progression directly or indirectly through negative regulators of the cell cycle such as the Rb protein. Moreover, it has been recently reported that PML could also be associated with another well-studied tumor suppressor protein, p53 [27-29]. This protein also functions as one of the important regulators of the G1 phase of the cell cycle. This finding provides further support on the regulatory effects of PML on the cell. However, the exact mechanism by which PML might affect the G1/S is still not understood. This requires investigation of the possible association of PML with the key regulators of the cell cycle, and a more careful analysis of the expression of these regulators in the presence of PML. We are currently studying the effect of PML on the promoter of a number of cell cycle regulators such as the dihydrofolate reudoctase (DHFR) and thymidine kinase.

Since EGFR functions as an important receptor, mediating the effects of EGF on cell growth, the inhibition of its activity may provide a mechanism for the growth suppressing effects of PML. In fact over-expression of EGFR is associated with several malignancies such as breast, colon and ovarian cancer [24-25]. It is therefore suggested that the *PML* gene could be considered as a potential agent in the therapy of these cancers.

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